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Interactions in Oncogenesis

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## **Introduction**

### **Nature of the problem/project**

In our original application, we proposed to study interactions between stromal and epithelial cells in development of tumors of the mammary gland (MG) in transgenic mice that express Shope growth factor (SGF). SGF is an epidermal growth factor (EGF)-like glycoprotein growth factor that elicits cellular proliferation and in virgin transgenic mice was associated with development of mammary gland tumors. We had found that SGF is produced mainly in stromal cells of the mammary gland (and other organs), and that proliferative and differentiative responsiveness to SGF were principally seen in the ductal and other epithelial cells of the mammary gland. Thus, we proposed to use this system to study how stromal and epithelial cell interactions produce preneoplasias and tumors in SGF transgenic mice.

### **Background of Previous Work by ourselves and others**

This background section will discuss (i) the roles of EGF-like growth factors (EGFs) in cell activation and growth; (ii) participation of EGFs in oncogenesis, particularly clinical tumor development in the human breast and experimental tumor development in the mammary gland; (iii) what is understood about the interactions between stromal and epithelial cells in oncogenesis; and (iv) tumor development in SGF transgenic mice. The first two subsections of this background reflect work performed by our and other laboratories. The final subsection describes work done on this system in our laboratory.

#### **(i). roles of EGF-like growth factors in cell activation, growth and oncogenesis**

There is a family of EGF-like growth factors (EGFs). EGFs stimulate responsive cells to proliferate and, sometimes, to differentiate (1). Therefore, EGFs play roles in cell proliferation and differentiation activities such as oncogenesis, wound healing and organ maturation.

SGF is a glycoprotein related to EGF (2). It is encoded by malignant fibroma virus (MV), which produces malignant tumors of fibroblasts. Epithelial proliferation overlies fibromyxosarcomas in MV-infected animals, and is felt to represent the influence of SGF production by MV-infected cells. (3). When the SGF gene is deleted, MV's virulence is attenuated. Epithelial proliferation and tumor spread are diminished. Instead of dying uniformly, most animals survive (4).

To understand how SGF acts as a growth factor, free of other viral genes we produced transgenic mice that express SGF. In this setting SGF induces mammary differentiation, proliferative preneoplastic lesions, or invasive adenocarcinomas, depending on the promoter construct used and the animal's age when SGF expression begins. In these studies, we proposed to study how stromal and epithelial SGF secretion and responsiveness affects target cells and leads to neoplasia.

The family of EGFs includes EGF, transforming growth factor- $\alpha$  (TGF $\alpha$ ), amphiregulin, cripto, three poxviral products (SGF, vaccinia growth factor (VGF) and myxoma growth factor (MGF)) and the potential HER2/neu ligands gp30 and p175. These all differ in primary structure, but generally share a constrained tertiary structure characterized by 3 overlapping disulfide bonds (5). Of these growth factors, EGF and TGF $\alpha$  are the best understood. EGF and TGF $\alpha$  are 53 and 50 amino acids (aa) respectively, and are produced by cleavage of larger pre-

cursors (6). The TGF $\alpha$  precursor may be glycosylated and anchored at the cell membrane, but its post-translational modifications are lost when the secreted form is cleaved from its membrane bound precursor (7). Thus, secreted forms of EGF and TGF $\alpha$  are not glycosylated. The poxviral growth factors and gp30 are larger and are all glycosylated (8). SGF is encoded as an 80 aa polypeptide, then cleaved and glycosylated to 12-16 kDa (1,9,10).

The effects of most of this family on cells depend on interactions with EGF receptor (EGFR). EGF, VGF, SGF and TGF $\alpha$  all bind the extracytoplasmic region of EGFR at different sites (11-14). Human EGFR is a 170 kDa transmembrane glycoprotein that resembles the products of viral oncogene, *v-erbB* and the cellular protooncogene HER2/neu (15). GF-receptor interaction initiates a cascade of events that leads to cell division. When ligand binds EGFR, the receptor oligomerizes and its affinity for ligand increases (16). Cytoplasmic EGFR tyrosine kinase activity is activated, and adjacent receptor molecules trans-phosphorylate each others' cytoplasmic domains (17).

Activated EGFR mobilizes a series of intracellular responses. Many enzymes associate with the cytoplasmic domain of EGFR via SH2 (src-homology-2) domains that bind activated EGFR phosphotyrosine, e.g., PI3 kinase, GTPase activating protein and phospholipase C $\gamma$ 1 (PLC $\gamma$ 1, 18-20). These enzymes are substrates for EGFR kinase activity. They are activated on phosphorylation by EGFR, and carry the message of growth factor-induced cellular stimulation to the cellular activation apparatus beyond. This leads to activation of PLC $\gamma$ 1, followed by hydrolysis of phosphatidyl inositol phosphates into inositol phosphates, which increase intracellular Ca (21,22). Ras protein is also activated via intermediate proteins, Grb2 and Sos1 (23-25). Ras activation is associated with phosphorylation/activation of raf1 kinase, which leads to activation of MAP kinases (26,27), then of jun-fos AP1 transcription factor. The latter causes increased gene transcription and leads to cell division (28,29).

#### (ii) participation of EGFs in oncogenesis, particularly mammary and breast oncogenesis

The EGF group of cytokines is important in both organogenesis and oncogenesis. They are needed for normal development and differentiation of many organs, including kidney, GI tract, lung and breast. EGF or TGF $\alpha$  stimulate normal mammary ductal growth, even in the absence of steroids, and are vital to mammary gland differentiation (30-32). We have found that SGF may induce mammary tumors or differentiation in transgenic mice, depending on the timing of its induction. A similar observation has been made for gp30 (33).

Tumor development is also linked to signal transduction via EGFR. EGF, TGF $\alpha$  and EGFR appear to be important for tumorigenesis in several organs via an autocrine loop: tumors make EGF and/or TGF $\alpha$ , plus high levels of EGFR. Tumor cells make EGFR and an EGFR ligand grow more slowly when the ligand is removed with anti-GF antibody (34). Anti-sense RNA that blocks EGFR expression reportedly suppresses transformation (35).

EGF alone does not elicit phenotypic transformation, but it may do so in concert with other agents (36). The *v-erbB* protein may transform cells precisely because it lacks ligand binding sites. This deficiency allows *v-erbB* protein to be constitutively activated in the absence of ligand (37). In addition, cells with high concentrations of EGFR may become phenotypically transformed in response to low concentrations of EGF (38).

The roles of EGFR and HER2/neu in breast cancer have been studied extensively (39). Several investigators have reported that EGFR+ tumors make and/or respond to members of the EGF family (40,41). In addition, EGFR expression is associated with aggressive behavior and

poor prognosis. Tumors that produce EGFR (EGFR+) are of higher grade and stage than EGFR- tumors, and express less estrogen receptor. EGFR+ tumors are also more highly proliferative and aneuploid (40). They tend to recur and kill patients more than EGFR- tumors (42). Detectable EGFR may be the most accurate predictor of survival (43). *C-erbB* (HER2/neu) protein is an EGFR-like receptor, whose expression has also been associated with poor prognosis in breast and other tumors (44).

A better understanding of the roles of EGFs in oncogenesis has come from the study of transgenic animals expressing EGFs. TGF $\alpha$  induces mammary hyperplasia, and in some cases, differentiation (alveolarization). Aged virgin TGF $\alpha$ -mice mainly showed atypical hyperplasias, while aged multiparous mice may develop secretory tumors (32,45,46). MG tumors are reported in transgenic mice expressing *c-myc*, *c-erbB2/neu*, *int-1*, *int-3* and *Ha-ras*, and in F1 hybrid mice transgenic for both *wnt-1* and TGF $\alpha$  (47,48).

The most common mammary preneoplasias are hyperplastic alveolar nodules (HAN) and ductal hyperplasia (DH). These are usually induced by mouse mammary tumor virus (MMTV), chemical carcinogens, hormonal stimulation or radiation (49-52). HAN and DH cells are immortal populations. That is, they can be serially transplanted indefinitely. On transplantation, these preneoplasias show hyperplastic growth patterns and are at high risk for neoplastic transformation (53,54). Studies proposed here will help to define the progression of preneoplasias into tumors and the effects of SGF expressed as a transgene.

We proposed to study transgenic mice expressing SGF, an EGF-like growth factor, as a model of mammary oncogenesis and differentiation. As indicated, we had collaborated to generate transgenic mice in which SGF expression was controlled by either an inducible promoter or a constitutive promoter. Our choices in this case were the metallothionein (MT) promoter and the constitutive Rous sarcoma virus LTR promoter (RSV-LTR, 55,56). RSV-LTR is a strong, constitutively active regulator. MT is substantially inducible by heavy metal (Zn).

We had reported in our first studies that early generations of virgin RSV-SGF transgenic mice showed marked preneoplastic MG ductal proliferation by 6 mo. By 8 mo., 1/3 of these had developed adenocarcinomas. In contrast, virgin MT-SGF mice that had been induced to express SGF at 2 mo. of age, showed MG differentiation without atypia. By *in situ* hybridization analysis of both sets of transgenic mice, we found that SGF was mainly expressed in mammary stroma, although the observed changes in the mammary gland histologic appearances were all epithelial. These observations suggested that since mouse mammary neoplasias and preneoplasias are typically readily cultured and are transplantable *in vivo*, SGF-mice might represent a model system to examine stromal-epithelial interactions in GF-related oncogenesis.

### (iii) what is known about interactions between stromal and epithelial cells in oncogenesis

A peculiar strength of this model is the opportunity it provides to study epithelial responsiveness to growth stimulation by stromal cells. Much evidence implicates interactions between breast stroma and epithelium in the growth of malignant tumors. This interaction involves secretion of and responses to insulin-like growth factors-I and -II (IGF-I, IGF-II). IGF-I and -II have different cell membrane receptors, though responses to IGF-II may be mediated through IGF-I receptor (57-59). Breast cancer cell lines usually respond to both cytokines but do not produce IGF-I. IGF-I is, instead, elaborated by mammary stroma adjacent to the tumor. Thus, IGF-I is a paracrine growth factor for breast tumor cells (60-62). IGF-II is also produced

by breast stroma, but is also made by some tumor cell lines. IGF-II, then, acts in both paracrine and autocrine fashions (63).

The interaction between SGF expressed as a transgene and target mammary epithelium resembles these reported data on the IGF's. SGF transgenic mice develop mammary tumors. The growth factor is recognized by EGFR. SGF is expressed in both epithelium and stroma, mainly in the stroma. Therefore, by developing mammary epithelial cell lines from transgenic and normal mice, and then transplanting them into the opposite recipients, we had proposed an experimental model that will allow the study and dissection of mechanisms of stromal-epithelial interactions that promote and sustain tumor and mammary gland growth.

Human breast cancer is a complex disease or group of diseases, involving a variety of independent risk factors such as parity, family history, etc. (64). Understanding human breast tumor development thus requires a number of model systems. The proposed studies of SGF-related mammary carcinogenesis complement other systems of breast carcinogenesis. For example, SGF elicits preneoplasias and invasive tumors in young virgin mice. Multiple parity starting at an early age decreases risk for human breast cancer but increases breast tumors in TGF $\alpha$  transgenic mice (32). SGF transgenic mice should thus provide additional insight into mechanisms of development and progression of breast tumors, and supplement other experimental models of mammary carcinogenesis.

#### (iv) mammary preneoplasia and neoplasia in SGF transgenic mice

##### a. Constructs used to produce transgenic mice

SGF constructs were made by cloning the SGF gene into mMT-1, a plasmid construct containing the mouse metallothionein promoter, and pRSVcat as expression vectors (65,66). (Insert orientation was confirmed by DNA sequencing.) These plasmids use the MT promoter and Rous sarcoma virus long terminal repeat (RSV-LTR) respectively as regulators of gene expression. The molecular strategies that were used to make these constructs are described in detail in the appended reprint (67), and are not recapitulated here.

Transgenic mice were made by microinjection of promoter-SGF constructs into (C57Bl/6 x DBA/2)F1 [hereafter, BDF1] embryos using standard techniques. Animals were screened for carriage of SGF transgene by assaying tail DNA. Two founder mice (#8, #9) carried SGF, and both had 3-5 copies of the gene. These mice were backcrossed to normal BDF1's. Offspring were examined for transgene carriage, and lines established from positive animals by sibling mating. We identified 3 founder mice carrying RSV-SGF and established lines from them in the same way.

In our earlier studies, we described generations of mice that were derived from the founders and early backcrosses. Backcrosses to normal parental strain mice and brother-sister matings had produced lines of transgenic mice. Because these were early generations, there were a substantial number of heterozygotes in these crosses. Brother-sister mating at that time produced a substantial percentage of offspring that did not carry the transgene. By this time, however, this does not happen: all matings produce transgenic mice. Although the apparent intensity of dot-blot signals is not always uniform, it is clear at this point that, unlike earlier generations, very few if any heterozygous transgenic mice remain in the lines of pure SGF transgenic animals.

Thus, 5 lines carry the SGF transgene, 2 with SGF controlled by the MT promoter, and 3 with SGF expressed constitutively under the control of RSV-LTR. The next section describes



our clinical and pathologic observations in the early generations of both MT-SGF and RSV-SGF transgenic mice. These findings formed the basis of this application.

**b. Histologic, clinical and *in vitro* observations on these transgenic mice.**

Histologic findings in mammary glands of virgin transgenic mice are summarized in Table 1 for MT-SGF mice in which SGF expression was induced after sexual maturity, and in Table 2 for the RSV-SGF mice, expressing SGF constitutively. The histology on which these summaries are based is illustrated in ref. #67. RSV-SGF mice are the basis of this application; so this discussion will focus on the preneoplastic and malignant proliferations found in those animals. Other observations are described and illustrated in the accompanying reprint (67). Unless otherwise stated, all observations are made on histologic sections taken near the nipples of the abdominal mammary glands.

**Table 1. MAMMARY HISTOLOGY IN VIRGIN MT-SGF TRANSGENIC MICE EXPRESSING SGF FOR 2 MONTHS**

<u>Construct</u>	<u>GF Expression</u>	<u>MG Histology</u>		
		<u>Ducts</u>	<u>Lobules</u>	<u>Other</u>
pMTSGF	Uninduced/2 mo.	Normal	None	Normal
pMTSGF	Uninduced/4 mo.	Normal	None	Normal
pMTSGF	Induced at 2 mo. for 2 months Observed at 4 mo. of age	Mild hyperplasia; Abundant protein- rich secretions in ducts, with +++ periductal fibroplasia	Development of lobules with protein- rich secretion	Normal

The findings from MT-SGF transgenic mice can be summarized as follows:

SGF expression elicits differentiation (alveolarization) and protein secretion in virgin mice when expression is begun at the age of sexual maturity for two months.

By contrast, RSV-SGF transgenic mice develop clear preneoplasia by 6 months of age and invasive secretory carcinomas by 8 months of age in 1/3 of RSV-SGF mice examined.

**Table 2. MAMMARY HISTOLOGY IN VIRGIN RSV-SGF TRANSGENIC MICE**

<u>Construct</u>	<u>GF Expression</u>	<u>Breast Histology</u>		
		<u>Ducts</u>	<u>Lobules</u>	<u>Other</u>
pRSGF	Constitutively expressed Observed at 2 months of age	Mild hyperplasia & atypia in ducts and ductules	None	Normal
pRSGF	Constitutively expressed Observed at 6 months of age	Highly abnormal. Marked hyperplasia extending through duct walls, into surrounding fat	None	Normal
pRSGF	Constitutively expressed Observed at 8 months of age	Highly abnormal. 1/3 of mice show invasive secretory adenocarcinoma	None	Normal

SGF has effects on other organs as well. These effects, which are not the subject of the current application, are described and illustrated in reference #67.

Production of and responsiveness to SGF by stroma and epithelium, and consequent cellular growth *in vitro* and *in vivo*, are important aspects of this application. We studied explanted fibroblasts from SGF transgenic mice *in vitro*. Skin fibroblasts from adult SGF mice transformed spontaneously in culture within 4 weeks: they lost contact inhibition, formed foci in monolayer culture and established colonies in soft agar. Control BDF1 fibroblasts invariably die within 6 weeks. Thus SGF in culture acts as a potent transforming agent.

Thus RSVSGF transgenic mice developed ductal hyperplasias, followed by occasional invasive carcinomas by 8 months of age. The oncogenic potential of SGF as it is produced by fibroblasts, are underscored by the rapid transformation of SGF transgenic fibroblasts *in vitro*.

#### c. Growth factor transgene expression

SGF transcription in the mammary gland was ascertained by RNA dot and Northern blot analyses, and *in situ* hybridization. SGF expression was studied in MTSGF mice  $\pm$  Zn for 2 mo., and in RSVSGF mice. MT-SGF and RSV-SGF MG, but not control MG, made mRNA that hybridized with SGF probe (See ref. #67 for *in situ* hybridization data and Northern analysis.)

Cellular patterns of transgene expression were studied by *in situ* hybridization (ISH) using SGF DNA as a probe. SGF DNA incorporating biotinylated dUTP (Boehringer-Mannheim) was hybridized to MG tissue sections from MTSGF, RSVSGF and normal BDF1 mice. This was followed by avidin, then biotin-alkaline phosphatase, according to established protocols (68). SGF transcript was detected in epithelial and stromal cells of many organs in both transgenic lines, but is expressed most strongly in vascular endothelium and other connective tissue cells, and MG epithelium.

SGF is expressed throughout the body in SGF-transgenic mice, in both stromal and epithelial cells. In the mammary gland SGF is mainly expressed by stromal cells.

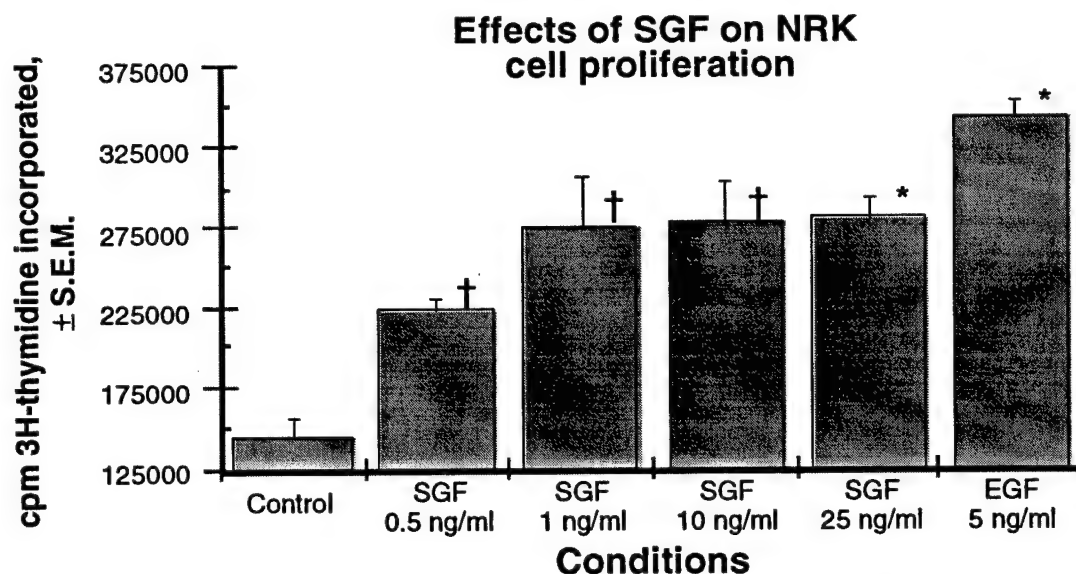
#### (d) Production and characterization of recombinant SGF

We had proposed to make biologically active recombinant SGF in order to elicit antiserum against the biologically active form of SGF. Other investigators have reported little success in making active SGF, using either prokaryotic expression (2), or chemical synthesis (14). To date, antibodies that can bind to SGF have not been reported. Antibodies vs. SGF peptides (A. Opgenorth, personal communication) or translation products made in *E. coli* do not recognize native SGF glycoprotein (2).

We have made recombinant glycoprotein SGF (rSGF) using baculovirus (AcMNPV, 69). Sf9 cells infected with SGF-AcMNPV produced a glycoprotein 12-16 kDa not found in Sf9 cells infected with wild type (wt) baculovirus, which matches published descriptions for SGF (2).

We reported that SGF produced in this fashion could be prepared from SDS-PAGE gels and used for functional studies in EGF-responsive cell lines (e.g., NRK cells). Such studies are illustrated in Fig. 1.

Figure 1 (following page). NRK proliferation stimulated by purified SGF or EGF. NRK cells were serum-starved overnight, and cultured for 4 days with SGF or EGF as shown. After 3 days 3H-thymidine was added. Cells were harvested one day later and incorporated radi-onucleotide counted. \*,  $P < 0.01$ ; †,  $P < 0.05$ , both compared to Control.



For all experiments described here, we will use recombinant SGF prepared by electroelution from SDS-PAGE of lysates of Sf9 cells infected with SGF-containing baculovirus.

Therefore, SGF produced from baculovirus yielded a glycoprotein similar in size to SGF from SFV-infected mammalian cells. This recombinant SGF has  $\approx 75\%$  of EGF's stimulatory activity. A chemically synthesized SGF peptide is reportedly 10% as active as EGF (14).

SGF is mainly expressed by the glandular stroma in mammary glands of transgenic mice. In this setting, its expression elicits mammary preneoplasia, leading to invasive carcinomas. We proposed in this grant to study tumor development using SGF transgenic mice and biologically active recombinant SGF as tools. This system may help to elucidate cellular, pathologic, and molecular mechanisms involved in mammary oncogenesis.

### **Purpose of the Present Work**

•It is the purpose of the work performed in this grant to study mammary oncogenesis in SGF-transgenic mice as it involves both production of growth factor and responsiveness to it. The studies performed in pursuit of this project are intended to determine the extent to which this requirement may be met by interactions between growth factor-producing stroma and -responsive epithelium.

### **Methods of Approach**

We proposed to use transgenic mice that express SGF as a transgene to study mammary oncogenesis. Cellular, biochemical and molecular parameters of oncogenesis in this system are to be defined, particularly as they relate to epithelial-stromal interactions. Thus, we would:

1. Define the natural history of mammary oncogenesis in SGF transgenic mice
2. Produce cell lines from preneoplasias and tumors from SGF mice, and characterize the growth characteristics of these cell lines
3. Define production of SGF in transgenic mice and study its induction of mammary neoplasias and differentiation
4. Assess stromal and epithelial interaction in GF production and responsiveness in the generation of mammary tumors and preneoplasias

We proposed to use SGF-transgenic mice to study interactions between mammary stromal and epithelial cells and mechanisms of growth factor-related oncogenesis. The experimental approaches proposed, the methods to be applied, and the time frames in which these studies were proposed to be completed are as follows:

1. Define natural history of mammary oncogenesis in SGF transgenic mice (months 1-24)

(a) Transgenic mice will be mated and left unmated to determine the natural history of SGF effects on the mammary gland following induction of SGF expression, from birth, in pregnancy and at different stages of development.

(b) Expression of recognized genetic markers associated with breast oncogenesis will be quantitated in RNA from mammary tissue.

2. Produce cell lines from preneoplasias and tumors from SGF mice, and characterize the growth characteristics of these cell lines (months 12-30)

(a) Cell lines will be established from ductal hyperplasias and tumors that arise in transgenic mice

(b) These cell lines will be studied for ligand binding by EGF receptor using Scatchard analysis

3. Define production of SGF in transgenic mice and study its induction of mammary neoplasias and differentiation (months 12-36)

(a) Antibody vs. SGF will be produced

(b) This anti-SGF antibody, in conjunction with cDNA probes, will be used to measure SGF production in the cultured transgenic fibroblasts and epithelial cells

(c) Immunohistochemistry and in situ hybridization will be used to localize SGF production and site of action within the mammary gland

4. Assess stromal and epithelial interaction in GF production and responsiveness in the generation of mammary tumors and preneoplasias (months 24-48)

(a) The ability of cells from transgenic mice to sustain their own growth will be measured by transplanting them into normal mice. The phenotypes of resultant proliferations will be studied by in situ hybridization and immunohistochemistry.

(b) Similarly, the ability of SGF-producing stroma to regulate oncogenesis will be determined by implanting normal cells into transgenic mice and assessing the outcome by in situ hybridization and immunohistochemistry.

## **Body**

The work done in the past year will be described with particular reference to the several tasks proposed in the original application. These have been recapitulated above. Experimental approaches were described at length in the original application and in our previous progress reports, and will not be repeated here, except to note changes that we have made in the original experimental plan. Some changes have been made in the direction of the project for several reasons. Mainly, we sought to accommodate new data from our own laboratory, and to understand the role of SGF in mammary gland biology in light of these changes. In addition, we sought to resolve experimental issues raised by new data.

1. Define natural history of mammary oncogenesis in SGF transgenic mice (months 1-24)

Our studies in the first years concentrated on generating the mice that were needed for these studies. This was done by breeding the several lines of SGF-transgenic mice, testing



them for transgene carriage, and grouping them for sacrifice at the time intervals specified in the original proposal.

(a) Transgenic mice were mated and left unmated to determine the natural history of SGF effects on the mammary gland following induction of SGF expression, from birth, in pregnancy and at different stages of development.

For this purpose, we bred transgenic mice from two different MT-SGF lineages and two different RSV-SGF lineages to provide sufficient numbers to begin our proposed systematic examination of the natural history of SGF-induced mammary gland epithelial proliferation. The proposed studies include the following:

- breeding and testing the SGF transgenic mice for transgene carriage. In the course of these studies, we decided that it was most advantageous to use only mice that were homozygous for SGF transgene carriage. Examination of heterozygotes would potentially complicate the analyses as we found that these mice occasionally express the transgene at lower levels than did homozygotes. Thus, substantial additional breeding was necessary to accommodate this need.

Nonetheless we have succeeded in breeding SGF transgenic mice to produce homozygous animals capable of expressing SGF.

- accumulation of sufficient numbers of transgenic mice to sacrifice the prescribed numbers of animals at the stated intervals (2 mo., 6 mo., etc.) and following the prescribed treatment regimens ( $Zn^{2+}$  treatment or control treatment for MT-SGF mice; pregnancy x0, x1, etc.). During the past two years, we had generated and cataloged sufficient numbers of the appropriate lines of mice both to allow breeding to continue, and to begin to sacrifice them in accordance with the proposed protocols.

Thus, we produced control (nontransgenic) mice that were sacrificed following 0 or  $\geq 2$  pregnancies. Mammary glands and other organs from these animals were saved for histologic examination, RNA extraction, etc., as proposed.

Furthermore we produced sufficient 2 month old virgin homozygous MT-SGF mice not fed  $Zn^{2+}$ , which have been sacrificed, again as previously proposed.

Along these lines, we have examined many mammary gland and other organ histologies from RSV-SGF, MT-SGF and normal mice. We have found that a significant percentage of SGF transgenic mice, mostly of the RSV-SGF lineage but occasionally of the MT-SGF lineages, show mammary gland proliferation and differentiation, even when they are virgins. The degree of mammary gland proliferation is highly advanced in some cases. In other cases, mammary differentiation includes milk production and extensive differentiation reminiscent of lactation.

(b) Expression of recognized genetic markers associated with breast oncogenesis were assessed in RNA from mammary tissue.

We have made RNA from mammary glands from many of the proposed groups of animals, and have accumulated the appropriate molecular probes with which to analyze these mice. The probes used are cDNA probes for c-myc,  $\beta$ -casein, whey acidic protein (WAP), retinoblastoma protein (Rb), p53, int-3, and Ha-ras, and oligonucleotide probes for  $\alpha$ -lactalbumin and gelsolin. All of the RSV-SGF animals proposed in the original application have been studied by these techniques, most have, and the results are summarized below. The analyses shown below are incorporated into a manuscript that is in preparation at this time. This Table is taken from that manuscript.

Previously pregnant mice were sacrificed 2 months following their last pregnancy (for 6 month previously pregnant mice) and 4 months following their last pregnancy (for 12 month previously pregnant mice). In the table shown below, the numbers of animals tested is indicated in parentheses.

Mice in the MT-SGF lines have all been generated, but a comprehensive analysis of these animals is not yet complete.

**Table 1. Gene expression in mammary RNA preparations from SGF-transgenic mice**

<u>Genotype(number) History</u>		<u>Gene tested</u>								
		<u>c-myc</u>	<u><math>\beta</math>-cas</u>	<u>WAP</u>	<u>Rb</u>	<u>p53</u>	<u><math>\alpha</math>-lac</u>	<u>gelsolin</u>	<u>int-3</u>	<u>H-ras</u>
<b><u>Control</u></b>										
2 month (2)	virgin	-	-	-	-	-	-	-	$\pm$	-
6 month (6)	virgin	$\pm$	$\pm$	-	-	-	-	-	+	-
6 month (6)	prev. preg.	++	++	-	-	-	-	-	+	-
12 month (6)	virgin	$\pm$	-	-	-	-	-	-	+	-
12 month (6)	prev. preg.	+	+/++	-	-	-	-	-	+	-
<b><u>Experimental</u></b>										
<b><u>RSV-SGF</u></b>										
2 month (2)	virgin	-	-	-	-	-	-	-	-	-
6 month (10)	virgin	+/++	+/++	+	-	-	-	-	+	-
6 month (6)	prev. preg.	+	+++	$\pm$ /+	-	-	-	-	+	-
12 month (8)	virgin	+/++	$\pm$	$\pm$	-	-	-	-	+	-
12 month (6)	prev. preg.	++++	+++++	++	-	-	-	-	+	-

The table shown above is remarkable for the following observations:

(1) Expression of c-myc in whole tissue homogenates is highly unusual, yet appears to be a hallmark of SGF transgene expression. Myc is also expressed at detectable levels in control pregnant mouse mammary glands.

(2) Even more striking is the association of SGF production with expression of differentiation-associated proteins,  $\beta$ -casein and whey acidic protein (WAP).

The lack of detection of  $\alpha$ -lactalbumin is of indeterminate significance, since in our hands the sensitivity of oligonucleotide probes is less than that of the cDNA probes used for most of the other cellular genes. (A cDNA probe for  $\alpha$ -lac was requested but not provided to us.)

A very extensive histologic analysis has been completed on these animals. The findings of this histologic analysis can be summarized as follows. Compared to control mice, approximately half of virgin RSV-SGF transgenic mice at 6 months of age show mammary glandular histologic changes consistent with early to mid pregnancy: alveolarization of the gland, secretory changes in the alveolar cells, and accumulated or inspissated proteinaceous material in the ducts of the mammary glands. By 12 months of age, the percentage of virgin mice that show these changes is much less.

However, in previously pregnant mice the changes induced by SGF in the mammary glands are striking. Lactational histology in these animals often persists for 4 months following weaning of their last litters. This lactational histology involves very extensive ductal and al-

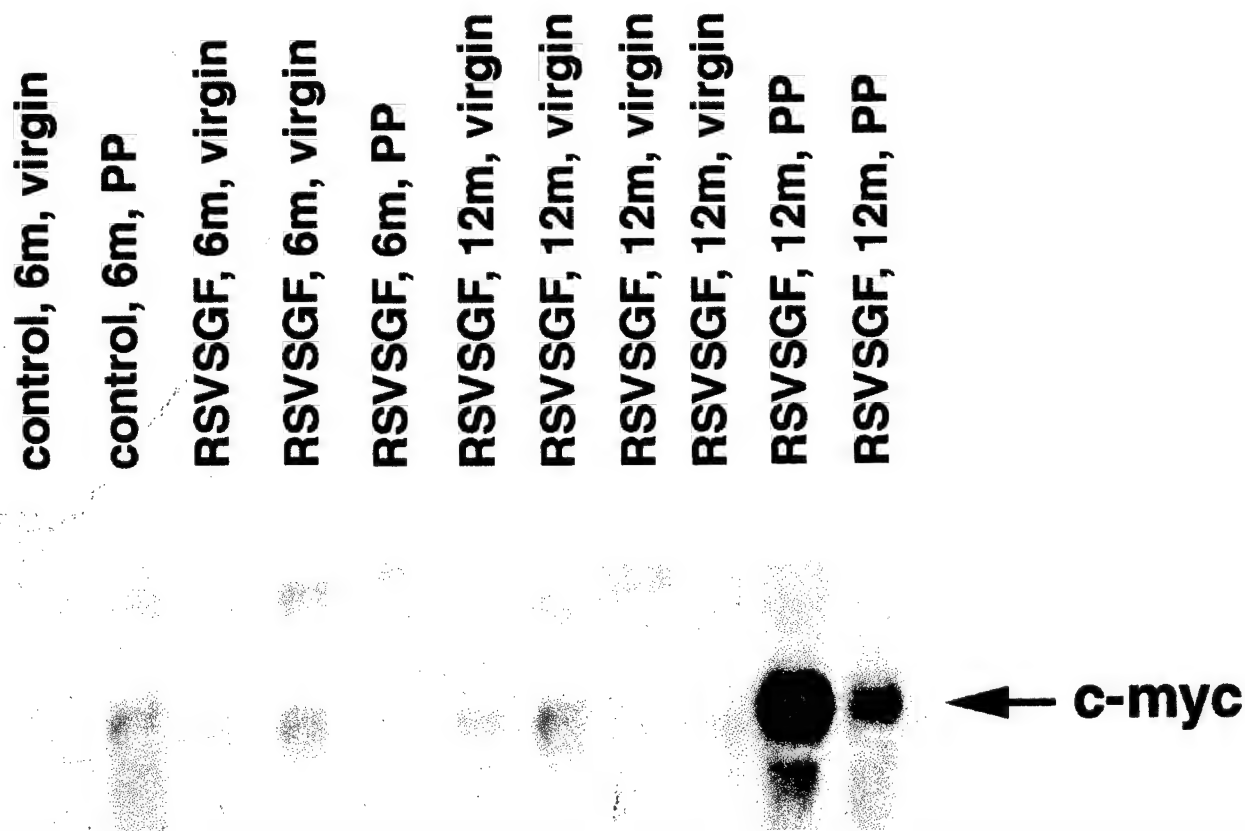
veolar proliferation, pronounced secretory cytologic changes in the alveolar cells, engorged alveoli and lobules, and dilated ducts. These glandular and ductal spaces contain abundant milk-like secretions. By contrast, control animals' mammary gland histologies are normal.

The animals in which histologic analysis showed these described profound differentiative effects of SGF were those in which the most remarkable increases in milk protein gene expression was demonstrated. Thus, it was in these mice that high levels (sometimes extremely high levels) of WAP and  $\beta$ -casein mRNAs were observed, and in which high levels of c-myc expression was also seen.

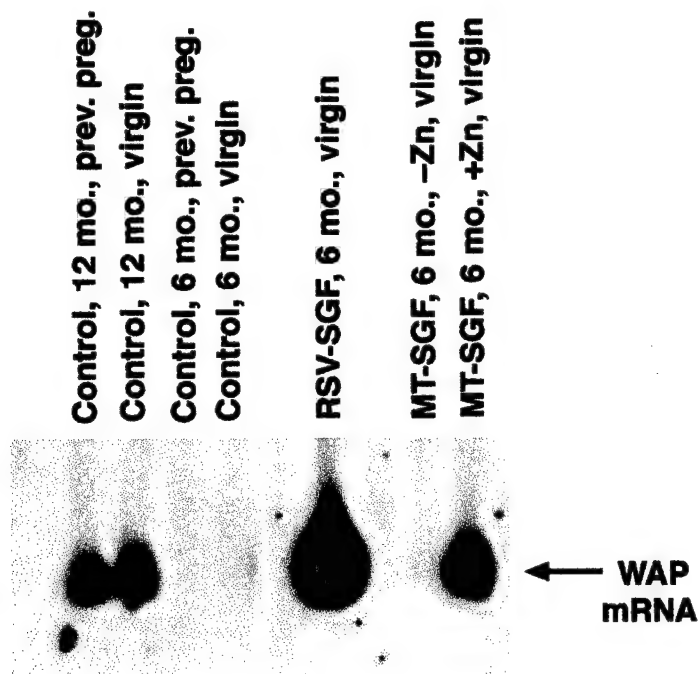
The most striking feature of these studies is the indication that SGF has a profound differentiative effect on mammary epithelial cells in RSV-SGF mice. That is, in some virgin mice and in many previously (remotely) pregnant mice, continued very high level expression of lactation-associated genes has been observed. This is in strong contrast to our earlier studies in which RSV-SGF mice showed ductal proliferation with substantial atypia, as well as some carcinomas. These findings suggest an effect of the increased inbreeding of the SGF mice on the phenotype displayed: as homozygosity approached 100%, the effect of the transgene tilted towards inducing differentiation rather than inducing proliferation.

Figs. 2. Illustrative Northern analyses of transgenic mice and control mice. As indicated above, 6 mo. old previously pregnant mice are 2 mo. post-pregnancy and 12 mo. old previously pregnant mice are 4 mo. post-pregnancy. The blots illustrated are for total mammary gland RNA preparations hybridized with cDNA probes for c-myc (a) whey acidic protein (b),  $\beta$ -casein (c) and WDNM1 (d), as indicated.

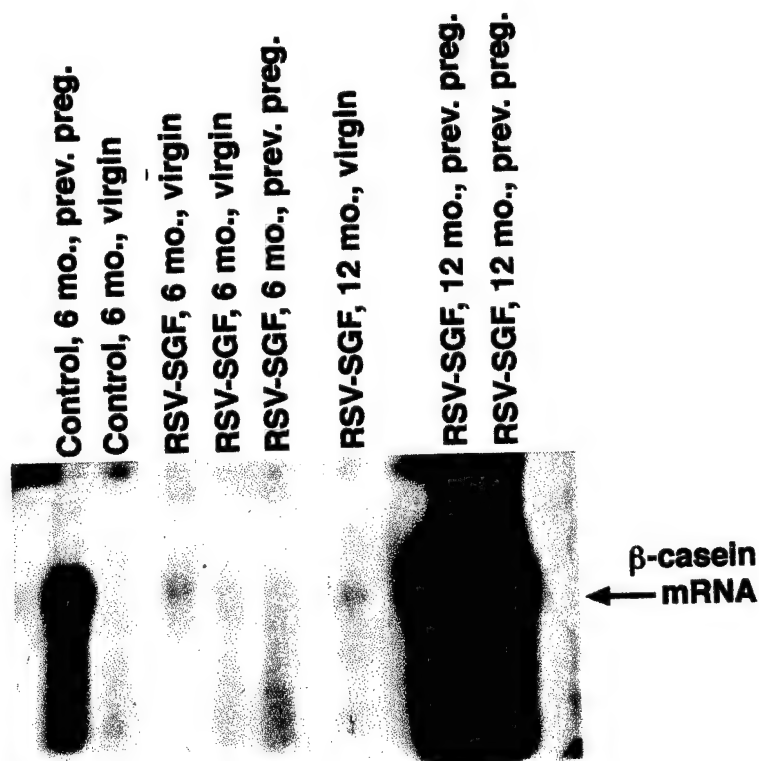
(a) Northern analysis of RSV-SGF transgenic mice and normal controls for expression of c-myc. Ages and pregnancy status are indicated. (PP indicates previously pregnant,  $\geq 2$  mo. prior to sacrifice.)



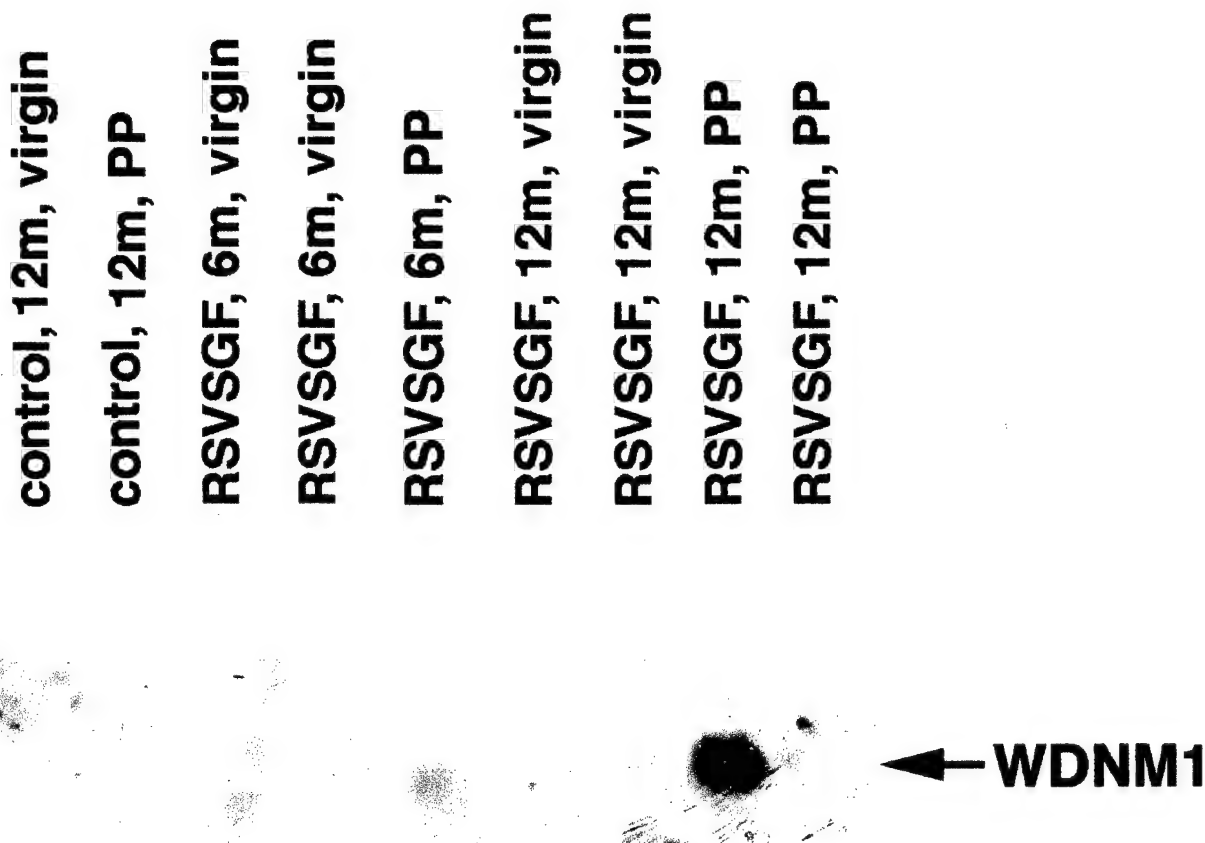
(b) Northern analysis of RSV-SGF transgenic mice, MT-SGF transgenic mice, and normal controls for expression of whey acidic protein (WAP). Ages and pregnancy status are indicated.



(c) Northern analysis of RSV-SGF transgenic mice and normal controls for expression of  $\beta$ -casein. Ages and pregnancy status are indicated.



(d) Northern analysis of RSV-SGF transgenic mice and normal controls for expression of WDNM1. Ages and pregnancy status are indicated.



In part because of the surprising directions taken by the analyses generated in the above studies, we began additional breeding to test to the possible effect(s) of combined SGF expression + abnormal p53 on the mammary gland of mice. These additional studies were occasioned in part by the observation that p53 mutation plays such an important role in human cancer, particularly human breast cancer (71-75). Therefore, in collaboration with Larry Donehower, Department of Molecular Virology, Baylor College of Medicine, we began breeding our SGF transgenic mice with p53-knockout mice that he had developed.

We began this breeding program shortly after the onset of the previous grant project. We received a pair of p53 -/+ mice, provided to us by Dr. Donehower. Our first goal with these animals was to produce a stock of p53 -/+ mice with which to breed the SGF mice. This was accomplished. We then identified both p53 -/- and p53 -/+ mice, and have crossed these animals with SGF mice of both the RSV-SGF and MT-SGF lineages.

The goal of establishing SGF+ p53 -/- mice, as described in the prior progress report, has proven more difficult than we had expected. We have made many p53 -/+, SGF+ mice of both sexes, and their phenotypes in terms of mammary histology and development appear to be indistinguishable from normal mice. Most of these mice are heterozygous for RSV-SGF, perhaps explaining the lack of a more SGF-like mammary gland phenotype. We have made a large

number of p53<sup>-/-</sup> mice, which have died at 6-9 months of age. Most of these deaths have been from lymphoma/leukemias. Deaths from osteosarcomas have also occurred. In addition, a number of mice that die spontaneously are consumed by cagemates and do not yield interpretable histologic findings.

We have, as well, noted a very strong preponderance of males among the SGF+p53<sup>-/-</sup> mice. That is, when pups are analyzed at 2-3 weeks of age, the male:female ratio among these animals is approximately 3:1. The poor fertility of the females of this genotype has further complicated our difficulties in establishing stable p53<sup>-/-</sup>-SGF<sup>+</sup> lines for analysis of mammary histologies. (Interestingly, the survival advantage of males during and/or shortly after embryogenesis is reversed in postnatal life. Among the p53<sup>-/-</sup>-SGF<sup>+</sup> mice females survive to 6-8 months far more readily than do males. The latter all appear to die within 4-6 months of birth.)

We are currently studying the reasons for the preponderance of males at birth in these animals, and have been in rather close contact with our collaborator in these studies, Dr. Donehower. Currently crosses are in progress to try to define any embryogenetic differences between male and female p53<sup>-/-</sup>-SGF<sup>+</sup> mice.

If our proposed lines of these combination transgenic-knockout mice can be developed, we will begin a systematic analysis of their mammary glands and other organs. Initially, the analysis of these animals will parallel analysis of the straight SGF transgenic animals described above. Subsequent analyses will depend upon the nature of the findings in our early studies.

## 2. Produce cell lines from preneoplasias and tumors from SGF mice, and characterize the growth characteristics of these cell lines

(a) Cell lines will be established from ductal hyperplasias and tumors that arise in transgenic mice

(b) These cell lines will be studied for ligand binding by EGF receptor using Scatchard analysis

Work on establishment of these cell lines is in progress. Dr. Pilarisetti has worked in the laboratory of Dr. Gilbert Smith (NCI) to learn the necessary procedures for culturing mammary epithelial cells.

At this point, we have successfully established stromal cell lines. These have been well enough established that aliquots have been frozen to preserve them for future analysis. These cells continue to be passaged. Progress has been somewhat slower in producing mammary epithelial cell lines, as several attempts have not yet yielded the expected results. We are currently continuing these studies.

## 3. Define production of SGF in transgenic mice and study its induction of mammary neoplasias and differentiation

(a) Antibody vs. SGF will be produced

(b) This anti-SGF antibody, in conjunction with cDNA probes, will be used to measure SGF production in the cultured transgenic fibroblasts and epithelial cells

(c) Immunohistochemistry and *in situ* hybridization will be used to localize SGF production and site of action within the mammary gland

We are working on this specific aim. (Specifically, Aims #3(a) and (c).) The first step in Aim (3a) was to produce anti-SGF antiserum. We have used SGF from our baculovirus system, and have immunized rabbits with 10-25 µg protein intravenously in saline every 3 weeks, bleeding one week after immunization. By Western blot, we have detected antibody activity. Therefore, we have developed antisera with antibody activity against SGF. Continued im-



munization and testing is being performed to develop sufficiently high anti-SGF activity for the second and third subaims of this specific aim.

With time, however, our stocks of productive recombinant baculovirus have produced increasingly poor yields of SGF. We have found that this is due to the loss of the SGF gene from the virus. Even when the original stock was recloned to produce a stock from the progeny of one SGF+ recombinant virus, we noted difficulties in producing adequate stocks of protein that could be applied effectively to these studies.

Consequently, we have reengineered our SGF-containing baculovirus to contain a his7 leader sequence. The purpose of this reengineering is to facilitate the identification and purification of the recombinant growth factor. We have succeeded in this endeavor, and are now producing this slightly modified SGF, which can be purified in one step using a Ni affinity column. The levels of SGF production achieved are relatively low, but the relative ease of separation of the recombinant glycoprotein and its preparation compensate in our minds for the low yields.

*In situ* hybridization studies have been applied to the analysis of SGF production in transgenic mammary glands and other tissues. We have found, as was previously suggested, that the expression of SGF in the mammary gland is principally a function of stromal cells. We are currently applying these approaches to our stromal cell lines.

We had proposed an extensive analysis of SGF production and localization by *in situ* hybridization, and these studies have largely been completed. We have, in addition, done an extensive analysis of expression of several of the key genes studied by Northern analysis, using *in situ* hybridization. Thus, *in situ* hybridization studies of the mammary glands using riboprobes for c-myc,  $\beta$ -casein and WAP have also largely been completed. Expression of these cellular genes in the epithelial cells of the mammary glands is striking, and correlates particularly with the Northern analyses and the histologic analysis (see above).

In addition to the expression of SGF in mammary stroma and epithelium, we have found that epithelia of a number of organs do support SGF expression, however. We have found that epithelium of kidney, liver and gastrointestinal tract express SGF. The relative proportions of stromal: epithelial expression vary from organ to organ, however. The identification of c-myc,  $\beta$ -casein and WAP transcripts in virgin mammary glands from SGF transgenic mice has provided stimulus for us to expand our *in situ* hybridization studies to these genes as well.

A preliminary version of a manuscript we are in the process of preparing is appended. This is a working copy, and both is incomplete in terms of the finished, polished product and contains notes, additions, and corrections in the margins. Nonetheless, it is the result of some of the studies supported here, and provides a clear indication as to part of our progress.

This grant focuses on the effects of SGF on the mammary gland. In the course of the proposed studies, we expect to define the natural history of murine mammary tumor development as a function of SGF stimulation, and to categorize these effects according to current concepts of mammary development, differentiation and oncogenesis.

### **Conclusions**

Our studies to date have clearly indicated that with the development of homozygous transgene-expressing mice the mammary glandular phenotype has changed, and now strongly favors a differentiative or development-inducing activity on the part of the glycoprotein growth factor, SGF. Although these results are not entirely unanticipated, based on the known

ability of EGFs to elicit both cellular proliferation and glandular differentiation, they underscore the potential applicability of differentiation therapy to the treatment and prevention of tumors. We hope that further studies of these transgenic mice in the context of this grant will define this area more fully and allow us to determine whether this glycoprotein growth factor may eventually find clinical application as an inducer of cellular differentiation and so perhaps as a tumor inhibitor.



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## Induction of Mammary gland Differentiation by a viral Growth factor

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## ABSTRACT

Shope growth factor (SGF) is a poxviral member of the Epidermal growth factor-like growth factor family. Epidermal growth factor-related peptides appear to play an important role in proliferation and differentiation, including organogenesis and oncogenesis. To study the effect of SGF in mammary gland development and differentiation, transgenic mice were developed that express SGF, driven by the ~~R~~<sup>0</sup>us Sarcoma Virus LTR and were examined for the mammary gland histology and expression of oncogenes and milk protein genes that are felt to be important in normal mammary gland growth, differentiation and tumorigenesis. Mammary gland from female transgenic and non-transgenic mice at different stages of development, virgins ( 2, 6 and 12 months ) and previously pregnant ( 6 and 12 months ) were tested for the expression of c-myc, int-3, H-ras,  $\beta$ -casein, WAP, p53 and Rb by northern blot analysis and localized expression by in situ hybridization.

Northern blot analysis indicates expression of c-myc which is generally associated with normal and malignant growth, showed 20-30 fold expression in 12 month previously pregnant transgenic mice compared to the 2 and 6m virgins and previously pregnant transgenic and non-transgenic mice. Low mRNA levels of  $\beta$ -casein, the major mouse protein milk was found in some of the 6 and 12 month virgin transgenic lines but their expression dramatically increased to about 50-100 fold in 12 month previously pregnant

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transgenic mice. Moderate expression of (WAP, <sup>WAP</sup>~~another milk protein gene~~) that is important in terminal differentiation and development of mammary alveolar cells were observed only in some of the 6 and 12 month virgins and previously pregnant transgenic mice. Steady level of int-3 expression was found in all the transgenic and non-transgenic mice. (None of the mice under study showed detectable expression of H-ras or of the tumor suppressor genes ( p53 and Rb). Interestingly, histology analysis revealed that all 12m previously pregnant transgenic mice was associated with extensive mammary gland differentiation and some of the 6 and 12 month virgin transgenic mice which expressed  $\beta$ -casein and WAP mRNA showed moderate differentiation. Furthermore, the principal site of transgene expression was found in the stromal cells of the mammary gland. Finally, these results together with significant expression of c-myc and  $\beta$ -casein and extensive differentiation in 12 month previously pregnant mice, suggests role of SGF in differentiating mammary gland.



## INTRODUCTION

Epidermal growth factor (EGF) like peptides, that are structurally related to EGF includes <sup>transforming</sup> TGF $\alpha$ , <sup>receptor</sup> cripto (CR), amphiregulin (AR), heparin-binding growth factor and 3 known poxviral glycoproteins ( Shope growth factor SGF, Vaccinia growth factor VGF and Myxoma growth factor <sup>which</sup> MGF ) have been implicated in participating in process ~~that include~~ cellular proliferation and differentiation ( Burgess A.W, 1989, Prigeant and Lemoine, 1992, Snedekar et al, 1991, Smith et al, 1995b ) and are also important regulators of many different types of normal and neoplastic cells ( Lyons and Moses, 1990, Normanno et al , 1993, 1994 b ).

Of these, most is known about EGF and TGF $\alpha$ , which stimulate normal mammary duct growth in the absence of steroids and are vital to mammary differentiation ( Carpenter and Cohen, 1979 ). TGF $\alpha$  was identified in the mouse mammary gland (Rall et al ., 1985, Brown et al , 1989, Liscia et al ., 1990, Snedekar et al., 1991) and also acts as an oncogene in vivo and appears to predispose mammary epithelium to neoplasia and carcinoma (Matsu et al ., 1990 ). Overexpression of TGF $\alpha$  in the mammary gland using mouse metallothionein promoter or the mouse mammary tumor virus (MMTV) promoter/ enhancer caused mammary carcinomas after a relatively long latency period of 7-12 months. Mammary carcinoma were stochastic and arose in predominantly female mice that had undergone multiple pregnancies ( Jhappan et al 1990, Matsui et al 1990 and Sandgren et al 1990 ). Moreover, multiple pregnancies potentiate and may be required for oncogenesis ( Sandgren, 1990 ).

Other members of EGF family of proteins includes Amphiregulin (AR) and Cripto (CR) (Ciccodicola et al, 1989, Plowman et al, 1990) which has been characterized as autocrine growth regulators in non-transformed human mammary epithelial cells (Kenney et al., 1993, Normanno et al . 1994 ). AR stimulates fibroblast growth and is often upregulated in breast cancer (Le Jeune et al ., 1993 ). Recent evidence also suggests a role for CR -1 and amphiregulin in early normal mammary development ( Kenney et al ., 1995). Considerable attention has been given to the closely related peptides that constitute the EGF like growth factor family, little is known about the activities and role of poxviral members ( SGF, MGF and VGF ) in mammary gland development. The Poxviral GF are substantially larger than EGF and EGF  $\alpha$  and are all glycosylated. SGF is a glycoprotein cleaved to 8KDa ( Prigeant and Lemoine 1992 ). SGF is encoded by malignant fibroma virus (MV), a poxvirus that produces disseminated proliferations of fibroblasts and epithelial hyperplasia (Strayer et al 1983). Inactivating the Shope growth factor gene, produced attenuated MV virulence and oncogenicity (Opgenarth et al 1992). SGF and MGF are encoded by several tumor-causing poxvirus as VGF is encoded by vaccinia virus, which does not induce tumors (Chang et al 1990 ). Unlike VGF, SGF and MGF are not known to be secreted ( Chang et al., 1990 ).

- Members of the EGF family binds to the EGF receptor and activate its intrinsic tyrosine kinase activity ( Massague, 1990, Salomon et al ., 1990 ), although native SGF and MGF have not yet studied, an SGF peptide appears to bind

EGFR as well ( Lin et al., 1988 ). SGF encodes a protein of 80 amino acids and shares 33-45% sequence homology with the EGF ( Chang et al., 1987 ). Our previous studies reported SGF expression is associated with a range of histologic abnormalities in mammary glands ranging from preneoplastic mammary ductal proliferation to occasional adenomacarcinomas in SGF transgenic mice. In addition, developed severe epithelial atypia in the stomach and papillary gastric tumors ( Strayer et al., 1993 ).

As mammary gland provides excellent system to study protein involved in organogenesis, cell differentiation and tumorigenesis, and since postnally the morphogenetic events in mammary gland are accompanied by cellular differentiation process, leading to the development of secretory epithelial cells which are capable of synthesizing and secreting milk proteins such as  $\beta$ -casein, WAP,  $\alpha$  lactalbumin and WDNMI ( Robinson et al 1995 ). In addition, tumor epidemiology suggests that malignant cell transformation is a multistep process that may involve different genetic and epigenetic changes ( Land et al., 1983, Klein and Klein, 1985 ). Among the genes which have implicated in mammary tumorigenesis either because their endogenous gene expression has been altered are c-myc ( Bonilla et al., 1988, Escotet et al., 1986, Borg et al., 1992 ) int-3 ( Robinson et al., 1992, Smith et al., 1995, Gallaham et al., 1996 ) H-ras ( Kumar et al., 1990, Miyamoto et al., 1990 ) p53 ( Levine et al 1991, Cultta and Koshland 1994 ) and Rb ( Weinberg, 1990, Goodrich and Lee, 1993 ).

mammary gland as a model system for studying the role of genes in organogenesis and tumorigenesis. The expression of milk proteins such as  $\beta$ -casein, WAP,  $\alpha$  lactalbumin and WDNMI is regulated by a complex network of genes and hormones. The expression of these genes is altered in mammary tumors, suggesting that these genes play a role in the development of mammary tumors.

To better understand the biological activities of SGF, we examined the effect of SGF in the mammary glands of mice expressing an SGF transgene under

RSV-LTR promoter. To further correlate differentiation with the current mouse mammary gland biology, we studied the pattern of expression of protooncogenes ( c-myc, int-3 and H-ras ) tumor suppressor genes ( P53 and Rb ) and differentiation marker genes (  $\beta$  -casein and WAP ) at different ages in virgin and previously pregnant mice. Expression of SGF and above oncogenes were evaluated by histology and in situ hybridization.

## MATERIALS AND METHOD

(C57BL/6 X DBA) F1 females were used for these studies. The transgenic mice carrying SGF transgene (RSV-SGF) have been described earlier ( Strayer et al., 1993). Transgenic mice were generated, different lines of which that carried approximately 4 to 6 copies of the SGF transgene. Three lines were obtained that expressed the transgene and one was analyzed in detail. Mammary glands were taken from normal and transgenic mice at 2m, 6m and 12m virgin and previously pregnant mice. Previously virgin mice were mated and sacrificed 4 months after delivery to obtain 12 months previously pregnant mice, and 2 month after delivery to obtain 6 month previously pregnant. At least 2 to 6 virgin and previously pregnant mice were analyzed for each data point in these studies.

**DNA analysis** Genomic DNA was prepared from tails of 3 to 4 week old transgenic mice according to the standard protocol ( Palmiter et al., 1982 ). Presence of the SGF gene was routinely detected by dot blot hybridization

**Probes:** Int-3 cDNA , was a generous gift from Dr. Gilbert Smith , NIH. cDNA probes for SGF, murine c-myc, murine WAP, murine  $\beta$ - casein, human H-ras, human p53 and human Rb were from our own lab.

**Northern Blotting :** Total RNA was extracted from the mammary gland using RNazol ( Cinna-BioTex, Inc, Friendswood, TX, ). 20 $\mu$ g aliquots of total RNA were separated by electrophoresis in 1% denaturing MOPS -formaldehyde- agarose gels, transfered to Hybond N<sup>+</sup> nylon membrane (Amersham International ). Integrity and concentrations of RNA were

verified prior to hybridization by methylene blue staining of transferred rRNA. The blots were hybridized to P32- radiolabeled ( $\sim 10^9$  cpm/ $\mu$ g) cDNA probes (Boehringer Mannheim) at 42°C overnight in 50% formamide. Filters were washed twice with 2X SSC, 0.1% SDS and 1X SSC, 0.1% SDS and twice with .1X SSC, 0.1% SDS at 45°C. The housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used for normalization of expression. The corresponding mRNAs were visualized by autoradiography.

**Histology** Thoracic Mammary tissues were excised from the transgenic mice at different ages, was fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections (8 $\mu$ ) were prepared and stained with hematoxylin and eosin. These fixed tissue specimens were also used for *in situ* hybridization studies.

**Preparation of cRNA probes** Digoxigenin - labeled antisense probes were prepared according to Boehringer Genius 4 kit from appropriately linearized plasmid. A 600bp cDNA sequence was used for SGF, 1900bp cDNA sequence for c-myc, 276bp cDNA sequence for  $\beta$ -casein and 450bp cDNA sequence for WAP. The cDNA sequences spanned the respective coding regions and they were cloned into PGEM vectors (Promega).

**In situ hybridization:** Paraffin-embedded mammary gland tissues were sectioned (8 $\mu$ ) and mounted on neoprene-coated slides and used for *in situ* hybridization studies. Sections were deparaffinized, treated with 20 $\mu$ g/ml proteinase K for 6-7 min at 37°C, postfixed in 4% paraformaldehyde and acetylated. cRNA probes (SGF, c-myc,  $\beta$ -casein and WAP) were hybridized overnight at 65°C in 50% formamide, 5x SSC, 1% SDS, 50  $\mu$ g/ml dextran

sulphate and 50 $\mu$ g/ml yeast tRNA. Washes were performed at 65°C in 50% formamide/ 2xSSC/ 1%SDS 2 times (30min) and 25% formamide/2xSSC / 1xPBS ( twice, 30min each ). After treating with 25 $\mu$ g/ml RNAase for 30 min at 37°C. DIG-labeled RNA probes were detected, after hybridization to target nucleic acids, by enzyme-linked immunoassay using an antibody-conjugate (anti-digoxigenin alkaline phosphatase conjugate, anti-DIG anti-AP). Finally the sections were subject to color was developed using Nitroblue tetrazolium and 5-Bromo-4-chloro-3-indolyl phosphate.

## RESULTS

**Histology of SGF- transgenic mouse mammary gland.** Mammary gland sections from the thoracic mammary glands of RSV-SGF transgenic mice showed abnormal differentiation of mammary tissues ( Fig. 1). This was true in mammary glands from both virgin and previously pregnant mice. Most virgin transgenic mouse mammary glands examined between 2 and 12 months were normal some show occasional ductal arborization and mild to moderate lobular alveolar development ( Fig 1a ). Proteinaceous material was seen in the ductal lumens (Fig 1b ). While the degree of differentiation differed from mouse to mouse, in general virgin mice between 2 and 6 month of age showed normal histology, while the most pronounced changes were seen in older animals.

Sections from mammary glands of RSV-SGF mice that had been pregnant previously but were at least 4 months postpartum almost uniformly showed highly abnormal degrees of persistent differentiation . These glands resembled lactating mammary tissues: they showed greatly increased mass of mammary tissue, extensive alveolarization, pronounced secretory activity by the cells within the alveoli, and large, dilated ducts filled with protein-rich secretions ( Fig 2 ). Histologically, therefore, these transgenic mice appeared to have retained a lactating histology many months after their single litter had been weaned.

Intermediate histologies were also seen in previously pregnant mice examined 4-6 months following delivery. ( Fig 3 ). These glands showed lactational differentiation that was less than described and illustrated above



for mice 2-4 months following delivery, but more than was seen in the mammary tissues of virgin mice.

No mice showed mammary carcinomas. Mammary ductal proliferation or atypia were not in these animals.

**Other tissues.** Other tissues examined histologically included the stomach, small intestine, large intestine, liver, spleen, pancreas, brain, lungs, heart, kidneys, skin, ovaries, uterus and testes. In general, the histologic features of these organs were similar to that reported previously ( Strayer et al., 1993 ). Gastric epithelial hyperplasia was noted, as was an increase in the size in the size and number of pancreatic islets , and in megakaryotes in the spleen, compared to non-transgenic mice of the same strain. However, the most prominent histologic feature observed in RSV-SGF transgenic mice was hepatocellular hyperplasia: bi- and multinucleate hepatocytes were increased in number, mitotic figures were increased, lobular architecture was disturbed ( Fig 4 ). These findings were very variable. They did not correlate clearly with the age of the mice, and were only seen in RSV-SGF transgenic mice. Neoplasia, either adenomatous or carcinomatous, was not seen in the livers of these animals.

### **Oncogene and milk gene expression**

To understand the effect of SGF on the mammary differentiation and oncogenesis, we analyzed the expression of different oncogenes ( c-myc, int-3, H-ras, p53 and Rb) and milk protein genes (  $\beta$ -casein and WAP ) at different ages in normal and transgenic mice carrying SGF transgene. At the time of parturition, genes encoding mammary -specific proteins like  $\beta$ -casein and WAP are fully active, and high levels of the corresponding RNA's are found

throughout lactation. Transcriptional induction of these genes during pregnancy occurs in a progressing differentiation of the alveolar mammary cell lineage ( Robinson et al., 1995 ). In addition, role of c-myc, H-ras, p53 and Rb has been implicated in mammary carcinogenesis.

Table 1 shows the pattern of expression of oncogenes and milk protein genes in mammary gland of virgin and previously pregnant mice at 2, 6 and 12 months of age as determined by Northern blotting. Transgenic (A) and Non-transgenic (B).

c-myc Northern blot analysis revealed steady levels of c-myc expression in the mammary gland all 2, 6 and 12 month both transgenic and non-transgenic virgin mice. Expression was highest in 12 month -old-previously pregnant mice: c-myc mRNA levels there were at least 30 fold above normal in these animals ( Fig. 5 ). Increased c-myc expression was not noted in 6 month previously transgenic mice.

$\beta$ -casein  $\beta$ - casein transcript was not detected in 2 month old transgenic and non-transgenic mice. However, low levels of expression were seen in 6m and 12 month virgin transgenic mice suggesting partial mammary gland differentiation. Interestingly, expression levels increased dramatically in 12 month old previously pregnant transgenic females compared to the non-transgenic mice. There was about 100- fold increase in levels of  $\beta$ -casein mRNA in 12 month previously pregnant transgenic mice, whereas 6m old previously transgenic mice showed modest increase in  $\beta$ -casein expression (Fig. 6). Histologica analysis ( see above ) correlated with these

findings: slight differentiation or secretion in those 6 and 12 month transgenic mice that showed low  $\beta$ -casein expression, while the virgin transgenic mice which did not show any detectable  $\beta$ -casein expression showed normal histology. Extensive differentiation in the mammary gland correlated with high level  $\beta$ -casein expression in 12 month previously pregnant mice. Thus these histologic and molecular analysis are concordance.

**WAP** Fig 7 shows the expression of WAP mRNA, another milk protein gene was seen only in transgenic mice. All the 12 month-old previously pregnant transgenic mice showed detectable, usually high levels of WAP mRNA, 2 of the 6m and 3 of 12 month virgin transgenic mice showed low levels of WAP mRNA which correlated with mildly to moderately increased differentiation on the histologic analysis. No transgenic mice showed any detectable WAP mRNA.

**H-ras , p53 and Rb** Expression of p53 and Rb ( tumor suppressor genes ) and H-ras expression was not detected in any transgenic and non-transgenic mice by northern blot analysis.

**Int-3** Mouse mammary tumor virus ( MMTV ) integration may occur at the int-3 locus activating it , by promoter insertion. This can yield a novel truncated 2.3 kb cellular int-3 mRNA initiated from within the viral 3' LTR sequence. This was not detected in any of the transgenic and non-transgenic mice screened. Steady levels of normal endogenous int-3 expression were detected in normal all mice.

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**Expression of SGF transgene in mammary tissues.** Expression of SGF was localized by *in situ* hybridization on mammary tissues. In general, SGF transcript was identified in RSV-SGF transgenic mammary glands in both stromal and epithelial cell types ( Fig. 8 ) . The predominant localization of SGF expression, however, was the mammary stromal cells. Stromal cells expressing SGF included fibroblasts, vascular endothelium, and adipocytes, as well as ( less prominently ) smooth muscle cells of the ductal walls and the vasculature.

**Expression of SGF in other tissues.** As was reported previously, SGF expression in RSV-SGF transgenic mice was seen widely in most organ systems ( not shown ). The pattern of stromal predominance seen in the mammary glands was not unique, but seen in other parenchymal organs as well.

**Table 1 Northern blot analysis of RNAs from transgenic mice RSV-SGF (A) and non-transgenic (B). Mammary gland from 2, 6 and 12 month virgin and previously pregnant (P) animals were excised, and RNA extracted. Whole RNA, 20 $\mu$ g was electrophoretically fractioned on 1% agarose, transferred to nylon membrane, then treated with  $^{32}$ P labelled DNA ( c-myc,  $\beta$ -casein, WAP, int-3, p53, Rb and H-ras ) under highly stringent conditions ( hybridization 50% formamide, 0.1% SDS, 42 $^{\circ}$ C; final washes, 0.1x SSC, 0.1% SDS, 45 $^{\circ}$ C). Hybridization was visualized by autoradiography. The + and - sign refer to the presence and absence of expression**

**A Transgenic mice**

Age	P/V	c-myc	$\beta$ -casein	WAP	int-3	P53	Rb	H-ras
2 m	V	---	---	---		---	---	---
2m	V	---	---	---		---	---	---
6m	V		---	---	+	---	---	---
6m	V	---	---	---	---	---	---	---
6m	V		---	---	---	---	---	---
6m	V		---	---	+	---	---	---
6m	V		+	---		---	---	---
6m	V	+	+	+		---	---	---
6m	V	++	---	---		---	---	---
6m	V	---	+	+	---	---	---	---
6m	V	+	---	---	+	---	---	---
6m	V	++	++	---	+	---	---	---
6m	P	+	+	+	---	---	---	---
6m	P	+	++	---	+	---	---	---
6m	P		++++	---		---	---	---
12m	V	+	+	---	+	---	---	---
12m	V	+	+	---	+	---	---	---
12m	V	+	+	---	+	---	---	---
12m	V	+	---	---	+	---	---	---
12m	V		---	---	+	---	---	---
12m	V	++	---	+	+	---	---	---
12m	V	+	---	+	+	---	---	---
12m	V	+	---	+				
12m	V	+	---					
12m	P	+++	++++	+	+	---	---	---
12m	P	++++	+++++	+	+	---	---	---
12m	P	++++	+++++	---	+			---
12m	P	++	++++	+	+			---

# **B Non-transgenic**

Age	P/V	c-myc	$\beta$ -casein	WAP	int-3	P53	Rb	H-ras
2m	V	+	---	---	---	---	---	---
2m	V	+	---	---	+	---	---	---
6m	V	+	---	---	+	---	---	---
6m	V	+	---	--	+	---	---	---
6m	P	++	++	---	+	---	---	---
6m	P	++	++	---	+	---	---	---
12m	V	++	---	---	+	---	---	---
12m	V	+	---	---	+	---	---	---
12m	P	++	++	---	+	---	---	---
12m	P	+	---	---	+	---	---	---